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THE ROLE OF VESICLES IN THE TRANSPORT OF FERRITIN THROUGH FROG ENDOTHELIUM

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SUMMARY

1. The transport of ferritin molecules by endothelial cell vesicles has been quantitatively investigated by electron microscopy. Single mesenteric capillaries of pithed frogs were perfused with solutions containing 6·7 g ferritin 100 ml.$^{-1}$ for known periods before fixation *in situ* with osmium tetroxide.

2. Two series of experiments were carried out: in the first series the perfusate contained bovine serum albumin (1·0 g 100 ml.$^{-1}$); in the second series the perfusate contained no protein other than the ferritin. To assess the molecular radius of ferritin in solution, the free diffusion coefficient of ferritin was measured in the presence and absence of albumin.

3. The free diffusion coefficient of ferritin in saline solution (110 m-mole l.$^{-1}$) was found to be $0{\cdot}35 \times 10^{-6}$ cm^2 sec^{-1} at 21 °C and was not affected by the presence of bovine serum albumin. This indicates that there is no significant binding of albumin to ferritin in solution and yields a value for the Stokes–Einstein radius of ferritin of 6·1 nm.

4. In all perfusion experiments the percentage of luminal vesicles containing ferritin exceeded the percentage of labelled cytoplasmic vesicles, which in turn exceeded the percentage of labelled abluminal vesicles.

5. Labelling of all vesicle populations was seen after perfusions lasting less than 1 sec. At this time luminal vesicles were more heavily labelled in the absence of albumin.

6. The labelling of luminal vesicles increased with lengthening perfusion times up to 30–40 sec, after which steady levels of labelling were achieved. The rate of rise in luminal labelling and the steady-state levels reached were both greater in the absence of albumin. By contrast cytoplasmic labelling increased above its initial value only after perfusions of longer than 10 sec.

7. In the steady state, labelled cytoplasmic vesicles contained, on average, fewer ferritin molecules than labelled luminal vesicles. This finding is inconsistent with translocation of labelled luminal vesicles across the cell.

8. It is suggested that the early constant labelling of cytoplasmic and abluminal vesicles is consistent with the existence of vesicular channels. Later cytoplasmic labelling may result from the transient fusion of cytoplasmic vesicles with labelled luminal vesicles for periods long enough to allow mixing of vesicular contents. Albumin may affect vesicular transport by its interaction with the endothelial glycocalyx.

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INTRODUCTION

The experiments described in this paper were designed to yield quantitative information about the passage of ferritin molecules through the vesicular system of endothelial cells. Since their discovery by Palade (1953) endothelial cell vesicles have been considered to be important in the transport of molecules through capillary walls. Much of the evidence for this has been qualitative (Palade, 1960; Jennings & Florey, 1967; Bruns & Palade, 1968; Simionescu, Simionescu & Palade, 1975; Johansson, 1978), and, when quantitative theories of vesicular transport have been developed, they have been based on morphometric data and physiological measurements of macromolecular permeability (e.g. Renkin, 1964, 1977). Recently, Loudon, Michel & White (1979) have investigated the passage of ferritin into the vesicles of frog endothelial cells by perfusing single mesenteric capillaries with ferritin solutions for known periods before fixing the tissue *in situ* for electron microscopy. Because their technique allowed them to measure the period of exposure of the endothelial cells to ferritin more accurately than had hitherto been possible, they were able to describe the development of a gradient of ferritin-labelled vesicles across the endothelial cell.

Loudon *et al.* devoted much attention to the labelling of vesicles opening on to the luminal surface of the endothelial cell and were led to suggest that a major diffusion barrier for ferritin might be present here. Their data on cytoplasmic labelling had some interesting features but were too few for firm conclusions to drawn. In the present paper we describe an extension to their experiments. Not only have we looked more extensively at the passage of ferritin molecules into the cytoplasmic and abluminal vesicles, but we have also carried out a parallel series of experiments where endothelial cells were exposed to ferritin in the absence of plasma proteins. It is known that the removal of plasma proteins greatly increases the permeability of frog capillaries to water and small hydrophilic molecules (Levick & Michel, 1973*b*; Mason, Curry & Michel, 1977) and it was speculated that circulating plasma proteins might reduce the entry of ferritin into luminal vesicles. To ensure that any effect of plasma proteins upon the entry of ferritin into vesicles was not due to the interaction of ferritin and albumin molecules in solution, the diffusion coefficient of ferritin was measured in saline solutions in the presence and absence of bovine serum albumin.

Preliminary reports of the data have been presented to the Physiological Society (Clough & Michel, 1979*a*, *b*).

METHODS

Solutions. Cadmium-free horse ferritin (Sigma Chemical Col., 10% (w/v) in 150 m-mole NaCl $l.^{-1}$) was diluted with distilled water to give a final ferritin concentration of 6·7 g 100 $ml.^{-1}$ in 100 m-mole $l.^{-1}$ NaCl. In experiments where albumin was also to be present in the perfusate, crystalline bovine serum albumin was added to the ferritin solution to give an albumin concentration of 1 g 100 $ml.^{-1}$. All solutions were filtered through a Millipore filter (pore size 0·65 μm) before use. The frog Ringer solution used had the following composition in m-mole $l.^{-1}$: NaCl, 111·0; KCl. 2·4; $MgSO_4 7H_2O$, 1·0; $CaCl_2$, 1·1; glucose, 5·5. The buffers used in the preparation of the tissue for electron microscopy (maleate, Caulfield's and cacodylate) were all diluted to 70% of their usual strength, to approximate in tonicity to frog tissue.

Animals and experimental procedure. Experiments were performed on the capillaries of the exteriorized mesentery of *Rana temporaria*, the brains of which had been destroyed by pithing. In experiments where no albumin was to be present in the capillary lumen, before perfusion with ferritin the frog's own circulation was replaced, via a cannula in the left aorta, with a cooled (13–18 °C) Ringer solution containing washed human red cells. The red cells were added to the perfusate so that the capillaries could be visualized and cannulated more easily. Perfusion pressures were between 25 and 30 cm H_2O. In other experiments the frog's own circulation was left intact until cannulating a chosen capillary and perfusing it with the ferritin solution.

Frogs were placed in a Perspex tray and a loop of intestine exteriorized through an incision in the lateral abdominal wall. The mesentery was draped over a small Perspex pillar and superfused with a cooled Ringer solution (13–17 °C), the temperature of which was continuously monitored by a thermocouple glued to the pillar. The tissue was transilluminated and viewed through a Wild M5 dissecting microscope, attached to which was a trinocular fitting for simultaneous observation and photomicroscopy. The procedure of cannulating and perfusion of single capillaries has been described previously (Michel, Baldwin & Levick, 1969; Levick & Michel, 1973*a*, *b*; Loudon *et al.* 1979). A chosen capillary, at least 400–500 μm in length, was cannulated with a glass micropipette of tip diameter 10–15 μm at a tip pressure of 1–2 cm H_2O. The pressure in the pipette was then adjusted to 30 cm H_2O and the vessel perfused with either a ferritin solution or with a solution of ferritin and 1 % bovine serum albumin. The perfusion was watched through the microscope and the time recorded with a stopwatch. The perfusion 'ended' when ice-cold osmium tetroxide (2 %) was dripped onto the tissue, although perfusion pressures were maintained for some 10–15 min following initial fixation. Photographs of the cannulated capillary were taken before, during and sometimes after fixation, and drawings were made to aid in later identification of the vessel (see Plate 1*A*).

Preparation of the mesentery for electron microscopy. Following initial fixation of the mesentery with osmium tetroxide, 6 % glutaraldehyde in cacodylate buffer was dripped onto the tissue *in situ* for a further 20–30 min. The mesentery was then dissected from the frog, immersed in glutaraldehyde for up to 12 hr and washed in maleate buffer. It was post-fixed in osmium tetroxide and, after further washing, stained *en bloc* with 2 % uranyl acetate in maleate buffer. The tissue was again washed in maleate buffer before dehydration by passage through a graded series of alcohols and embedding in Durcupan resin. The perfused capillary was identified in the block using the photographs and drawings made during perfusion and ultrathin sections (60–80 nm) cut with a Cambridge–Huxley microtome. The sections were mounted on uncoated copper grids and examined in an AE1 6EMB electron microscope at an accelerating voltage of 60 kV. Electron micrographs of the capillary endothelium were taken at a magnification of × 30,000 and the photographs enlarged to give working prints of magnification × 90,000.

Estimation of vesicular labelling with ferritin. The labelling of luminal, cytoplasmic and abluminal vesicles was assessed from electron micrographs and expressed as: (i) the fraction of vesicles in a given population containing one or more ferritin molecules, N_L/N_T; (ii) the mean number of ferritin molecules per vesicle, F/N_T; (iii) the mean number of ferritin molecules per labelled vesicle, F/N_L; where N_T is the total number of vesicles counted, N_L is the number labelled and F is the total number of ferritin molecules seen within the vesicles.

Usually over 100 vesicles were counted before a value for ferritin labelling was accepted. Values for labelling after certain perfusion times (0, 5, 15, 30, 240 and 270 sec) were based on data from several experiments. Here, the mean values were obtained by pooling N_T, N_L and F from all experiments for each vesicle population. The fraction of vesicle volume which lay outside the section was assessed to be 0·1875 (Loudon *et al.* 1979) and final values were adjusted for this.

Since N_L/N_T is a frequency, the 95 % confidence limits for the mean values of N_L/N_T were calculated as:

$$\pm 1{\cdot}96\sqrt{\left(\frac{N_L/N_T(1-N_L/N_T)}{N_T}\right)}.$$

Measurement of the diffusion coefficient of ferritin and calculation of molecular radius. Diffusion coefficients for ferritin were measured using a simplified form of the Fürth microdiffusion cell (Fürth, 1945; Levick & Michel, 1973*a*). A solution of ferritin (6·7 g 100 ml.$^{-1}$) in frog Ringer solution was placed in one half of the cell in contact with frog Ringer solution in the upper half. As the yellow ferritin diffused into the frog Ringer solution its progress was monitored by followed the changes in optical density through the cell. For a given (reference) concentration of ferritin, the relationship between distance moved and the square root of time is linear, with a slope, S, related to the diffusion coefficient, D_F, through the expression

$$D_F = S^2/4\{\mathrm{erf}^{-1}\,[1-2(c/c_1)]\}^2,$$

where c represents the reference concentration of ferritin and c_1 is the initial concentration of ferritin in the lower half of the diffusion cell.

To assess the effect of albumin on the rate of diffusion of ferritin in frog Ringer solution, the measurements were repeated after the addition of 1 % bovine serum albumin to both halves of the diffusion cell. The molecular radius of ferritin, a_{es}, was estimated from the relationship:

$$a_{es} = \frac{RT}{6D_F \pi\eta N},$$

where η is the viscosity of water at 21 °C (room temperature), RT are the gas constant and absolute temperature, and N is the number of molecules per mole (Avogadro's number).

RESULTS

The diffusion coefficient of ferritin in the presence and absence of albumin

In six experiments, the diffusion coefficient (D) of ferritin was estimated in Ringer solution. Each experiment allowed several independent estimates of D at different concentrations so that a total of twenty-eight independent estimates of D could be made for ferritin in the absence of albumin, together with sixteen estimates made in five experiments where ferritin diffused through a Ringer solution containing bovine serum albumin at a concentration of 1·0 g 100 ml.$^{-1}$. The mean value of D in the presence of albumin was 0·347 ($\pm$0·0096) $\times 10^{-6}$ cm^2 sec^{-1} ($n = 16$) and in the absence of albumin was 0·0355 ($\pm$0·0095) $\times 10^{-6}$ cm^2 sec^{-1} ($n = 28$). The Stokes–Einstein radius of ferritin calculated from these values of D is 6·19 $\pm$ 0·17 nm ($n = 28$) in the presence of albumin, 6·05 $\pm$ 0·17 nm ($n = 16$) in the absence of albumin and 6·12 $\pm$ 0·31 nm when based on the mean value of all forty-four estimates of D (0·353 $\pm$ 0·018 cm^2 sec^{-1} $\times 10^6$, $n = 44$). These data reveal that there is no significant interaction between ferritin and serum albumin molecules in Ringer solution. Furthermore, the estimates of Stokes–Einstein radius agree closely with other estimates of the molecular radius of ferritin (Hoare, Harrison & Hoy, 1975).

General morphology of vesicles in frog endothelial cells

General descriptions of the ultrastructure of frog mesenteric capillaries and their endothelial vesicles have been given by Mason, Curry, White & Michel (1979) and Loudon *et al.* (1979). Endothelial vesicles were classified as luminal, cytoplasmic and abluminal (Bruns & Palade, 1968; Loudon *et al.* 1979). Vesicles were said to be luminal or abluminal if their cavities communicated with the luminal or abluminal surface through a neck which could be seen in the electron micrograph. Vesicles were said to be cytoplasmic if their cavities appeared to be completely enclosed, irrespective of their proximity to either luminal or abluminal surfaces. Two measurements of diameter perpendicular to one another were made on each vesicle to estimate its mean diameter. Values for two samples of vesicles taken from capillaries perfused with albumin-free and albumin-containing solutions are given in Table 1. It is seen that there are no significant differences in dimensions of vesicles at different sites in the cell, nor does perfusion with albumin-free solution lead to a change in vesicle diameter. The values of vesicle diameter are similar to those reported by Loudon *et al.* (1979). The frequency of luminal, cytoplasmic and abluminal vesicles expressed as a fraction of the total vesicle population does differ from previous figures, but this may in part relate to our definition of luminal and abluminal vesicles.

TABLE 1. Diameter and frequency of vesicles in perfused frog mesenteric capillaries

	Luminal	Cytoplasmic	Abluminal
A. Perfusion with Ringer solution, 1 % albumin and ferritin (6·7 g 100 ml.$^{-1}$)			
Diameter (nm)	69·9	66·8	68·3
±S.E.M.	2·5	2·2	1·3
(*n*)	(115)	(145)	(78)
Frequency of total vesicle population	0·18	0·65	0·17
B. Perfusion with Ringer solution and ferritin (6·7 g 100 ml.$^{-1}$)			
Diameter (nm)	69·9	67·2	66·9
±S.E.M.	3·6	1·8	2·4
(*n*)	(156)	(103)	(81)
Frequency of total vesicle population	0·15	0·71	0·14

Mean diameter for the total population: 68·2±0·59 nm (n = 678).

Labelling the vesicle populations with ferritin

Figs. 1 and 2 show the fractions of each vesicle population labelled with one or more ferritin molecules after varying periods of perfusion with albumin-containing and albumin-free solutions. Three general features are conspicuous in both diagrams: (i) at all times there is a gradient of labelling across the endothelial cell, more luminal vesicles being labelled than cytoplasmic vesicles and more cytoplasmic vesicles being

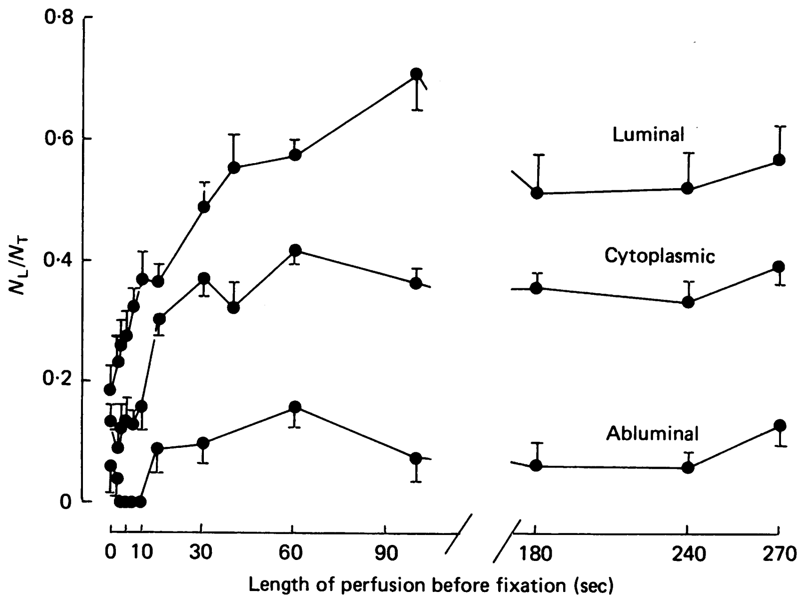

Fig. 1. The relationship between the fraction of luminal, cytoplasmic and abluminal vesicle populations labelled with ferritin molecules (N_L/N_T) and the period of perfusion with a ferritin solution (6·7 g 100 ml.$^{-1}$) containing 1 g 100 ml.$^{-1}$ bovine serum albumin, before fixation of the tissue. Values are corrected for sectioning losses. Each point represents the mean and 95 % confidence limits of a population of more than 100 vesicles taken from usually more than one experiment.

labelled than abluminal vesicles; (ii) some labelling is seen in all three populations at less than 1 sec (plotted as zero time); (iii) the levels of labelling appear to become steady after exposure to ferritin for 40–90 sec.

Closer inspection of Figs. 1 and 2 reveals that luminal N_L/N_T rises more slowly in the presence of serum albumin and attains a lower level of labelling in the steady state. The initial rate of rise of N_L/N_T is 0·016 sec^{-1} in the presence of albumin and 0·024 sec^{-1} in its absence. This effect of albumin on the initial rise of luminal N_L/N_T

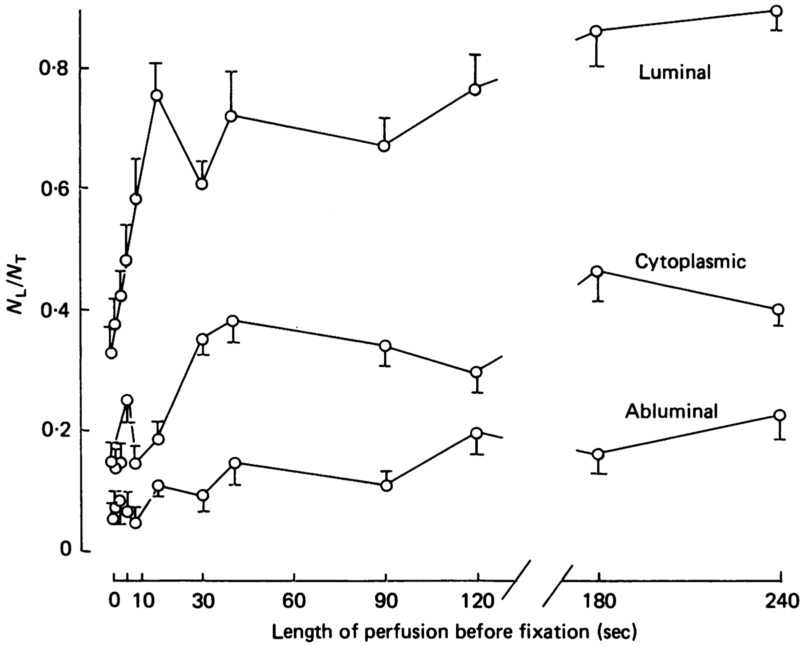

Fig. 2. The relationship between N_L/N_T for luminal, cytoplasmic and albuminal vesicles populations and the period of perfusion with a ferritin solution (6·7 g 100 $ml.^{-1}$) containing no albumin, before fixation. Values estimated as in Fig. 1.

is similar to its apparent effects on the initial and steady-state values of N_L/N_T. After exposure to ferritin for less than 1 sec (zero time), luminal N_L/N_T is 0·19 in the presence of albumin and 0·33 in its absence. At later times where luminal N_L/N_T appears to be steady, its value in the presence of albumin is 0·59 and in the absence of albumin 0·90.

The initial levels and rates of labelling in the presence and absence of albumin are shown more clearly in Fig. 3. This also reveals interesting differences between the early changes in N_L/N_T in the different vesicle populations. Whereas luminal N_L/N_T rises steadily from its initial value as exposure to ferritin increases, N_L/N_T for the cytoplasmic vesicles remains approximately constant. It is only at a time later than 10–15 sec that N_L/N_T begins to increase in the cytoplasmic and abluminal populations. That there is a genuine increase in the rate of labelling of cytoplasmic vesicles between 15 and 30 sec can be demonstrated by comparing the values of N_L/N_T at 0, 10, 15 and 30 sec. There is no significant difference between values of N_L/N_T at 0 and 10 sec in the presence of albumin, and 0 and 15 sec in the absence of albumin. There is, however, a highly significant difference in N_L/N_T between 10–15 and 30 sec in both the presence and the absence of albumin.

Both in the presence and in the absence of albumin, labelling of the abluminal vesicles is low. After perfusions of up to 10 sec, albuminal N_L/N_T varies between 0 and 5 % in the presence of albumin and maintains a value of around 5 % in the absence of albumin. At later times abluminal N_L/N_T rises to 10–15 % in the presence of albumin and 20–30 % in the absence of albumin.

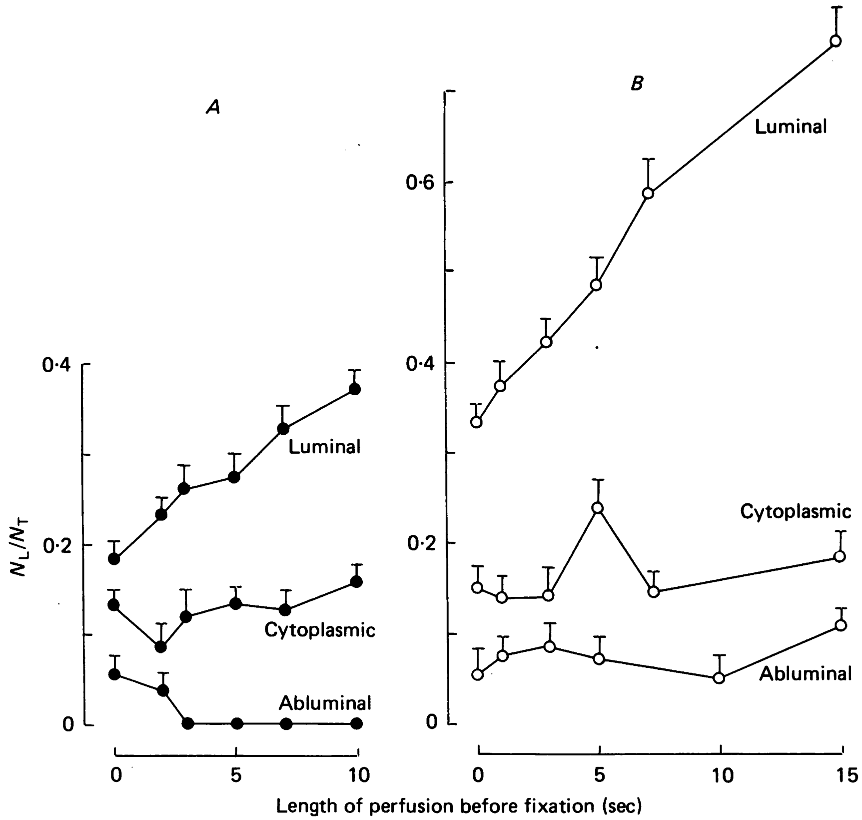

Fig. 3. A time-expanded plot of the relationship between N_L/N_T and time, for periods of perfusion of less than 15 sec. *A*, 1 % albumin in the perfusate; *B*, no albumin in perfusate. Values estimated as in Figs. 1 and 2.

The concentration of ferritin within the luminal vesicles: effects of albumin

Loudon *et al.* found the distribution of ferritin molecules in luminal vesicles could be described by a Poisson distribution. In Fig. 4*A* our data for the mean number of ferritin molecules for luminal (F/N_T) and the corresponding values of N_L/N_T for the luminal vesicles are compared with the relationship predicted by a Poisson distribution. The majority of points appear to fall below the curve and this is particularly true of the non-steady-state data taken from experiments where albumin was not present in the perfusate. Although these deviations are only significant at the 0·1 level. (Student's *t* test), they suggest that a fraction of the luminal vesicles is not available for labelling at earlier times. When only points from longer perfusions ($\geqslant$ 60 sec) are considered (Fig. 4*B*), they cluster around the Poisson curve, suggesting that this describes the steady-state distribution of ferritin molecules in the luminal vesicles. It is seen that F/N_T as well as N_L/N_T is increased in the absence of albumin.

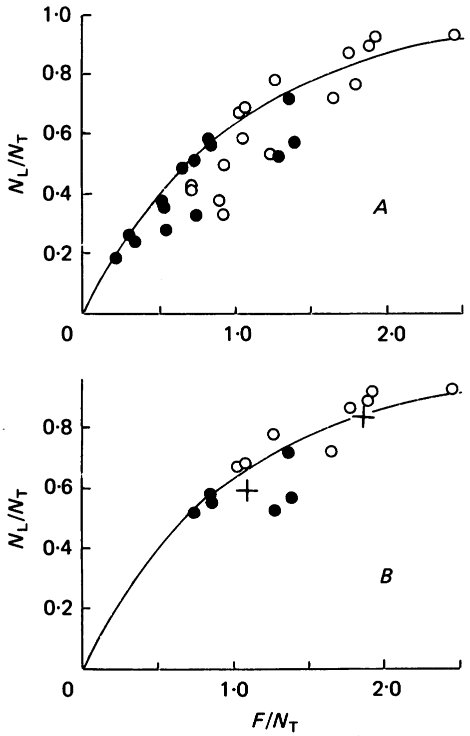

Fig. 4. The relationship between the fraction of the luminal vesicle population labelled with ferritin (N_L/N_T) and the mean number of ferritin molecules per luminal vesicle (F/N_T) for all experiments (*A*) and for experiments in which perfusion times exceeded 40 sec, where steady levels of labelling appeared to have been reached (*B*). Data corrected for sectioning losses. The smooth curve is the relationship predicted for a Poisson distribution. Open circles, no albumin in perfusate; filled circles, 1 % albumin in perfusate; crosses, means of open and filled circles in *B*.

Loudon *et al.* also compared the mean value for the number of ferritin molecules per labelled luminal vesicle (F/N_L), in the steady state, with a value predicted from the luminal concentration after allowance had been made for our data in Table 2. The steady-state values of F/N_L have been calculated in the presence and in the absence of albumin as:

$$\frac{\Sigma \text{ (ferritin molecules in all luminal vesicles in steady state)}}{\Sigma \text{ (labelled luminal vesicles in steady state)}},$$

Since N_L/N_T became relatively constant after 40 sec of perfusion, a steady state was regarded as being established at this time.

Table 2 shows that both in the presence and in the absence of albumin, F/N_L is very much lower than is predicted from the luminal concentration and the vesicular volume. Strictly speaking the predicted values should be compared with mean values of F/N_T, which reveals an even larger discrepancy.

The exclusion of ferritin from the luminal vesicles is increased by the presence of albumin in the perfusate. If the steady-state distribution of ferritin molecules in the luminal vesicles is described by a Poisson distribution, then it can readily be shown that F/N_L in the absence of albumin is significantly greater (at the 0·01 % level) than its value in the presence of albumin. Thus an interaction between serum albumin and the capillary wall, which is known to have a profound effect on capillary permeability to water and low molecular weight solutes (Mason *et al.* 1977; Levick & Michel, 1973*b*), also appears to affect the uptake of macromolecules into the vesicles.

TABLE 2. Ferritin contents of luminal vesicles (molecules per vesicle)

	Values predicted from lumen concentration		Observed values			
			Albumin present		Albumin absent	
	F/N_L	F/N_T	F/N_L	F/N_T	F/N_L	F/N_T
	6·9	6·9	1·79	1·046	2·22	1·862
No. of labelled vesicles counted ...			178		321	
Total vesicles counted ...			374		499	

Comparison of F/N_L or F/N_T in presence and absence of albumin, assuming each represent means of Poisson distributions of $n_1 = 374$ and $n_2 = 499$, indicate that F/N_L and F/N_T are greater in the absence of albumin ($P < 0{\cdot}002$).

Concentration of ferritin in luminal and cytoplasmic vesicles

In Fig. 5. values for F/N_L of the cytoplasmic vesicles are compared with values for F/N_L determined in the same experiment for the luminal vesicles. Experiments where the perfusion lasted for 15 sec or less have not been included in this comparison as other observations suggest a separation in the labelling of the luminal and cytoplasmic vesicles at earlier times. It is seen that at all times luminal F/N_L is greater than cytoplasmic F/N_L. That such a difference persists in the steady state suggests the labelled cytoplasmic vesicles are not translocated luminal vesicles.

Steady-state distribution of ferritin molecules in the labelled vesicles

Fig. 6*A*, *B* and *C* are histograms displaying the distribution of ferritin molecules in the luminal, cytoplasmic and abluminal vesicles respectively for all experiments both in the presence and absence of albumin. As expected from their higher F/N_L (Fig. 5) the luminal vesicles have a wider range of labelling than the cytoplasmic and abluminal vesicles. The cytoplasmic vesicles show a similar distribution of ferritin molecules but over a narrower range, whereas the abluminal vesicles are rarely labelled with more than one or two ferritin molecules. It is worth noting that the cytoplasmic and abluminal distribution suggest that they might arise as a result of dilution of the luminal distribution. There is no suggestion that the cytoplasmic labelling might be derived from two separate distributions.

DISCUSSION

The development of a gradient of ferritin-labelled vesicles in endothelial cells of capillaries perfused with ferritin solutions is direct evidence for the participation of vesicles in the transcapillary movement of ferritin. In demonstrating this we confirm the observations of Jennings & Florey (1967), Bruns & Palade (1968), Casley-Smith & Chin (1971) and Loudon *et al.* (1979) and reinforce the more general view of the involvement of vesicles in vascular permeability to macromolecules. A more detailed interpretation of our data, however, leads us to a novel role for vesicles in the transport process.

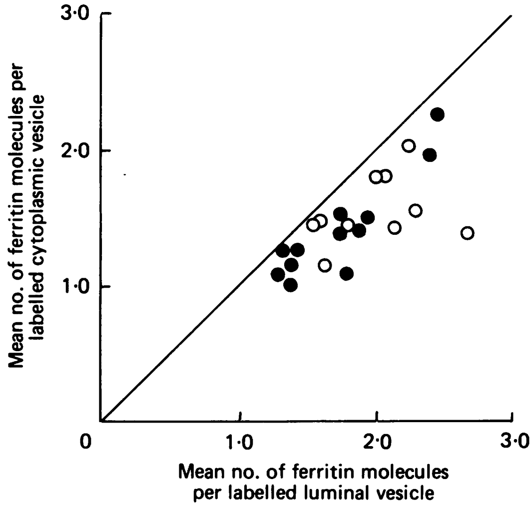

Fig. 5. A comparison of the mean number of ferritin molecules per labelled luminal vesicle and the mean number of ferritin molecules per labelled cytoplasmic vesicle for each experiment where perfusion time exceeded 15 sec. Open circles, no albumin in perfusate; filled circles, 1 % albumin in perfusate. Continuous line is the line of equality.

Vesicular transport by translocation or by transient vesicular fusions

The traditional view of vesicular transport (Palade, 1960; Mayerson, Wolfram, Shirley & Wasserman, 1960; Renkin, 1964) envisages vesicles at the luminal and abluminal surfaces, their contents equilibrated with the extracellular fluids, budding off to move to the opposite surface of the endothelial cell. Thus quanta of plasma and interstitial fluid are transported by vesicle translocation and it is assumed (Tomlin, 1969; Shea, Karnovsky & Bossert, 1969; Green & Casley-Smith, 1972) that equal numbers of vesicles move in opposite directions across the endothelial cell. Thus, at any time, half the cytoplasmic vesicles have just left the luminal surface and half have just left the abluminal surface.

For experiments such as ours where a steady concentration difference of ferritin is maintained across the endothelial cell, the hypothesis of vesicle translocation makes simple predictions about levels of cytoplasmic labelling which may be compared with our observations. If half the cytoplasmic vesicles are derived from the luminal surface

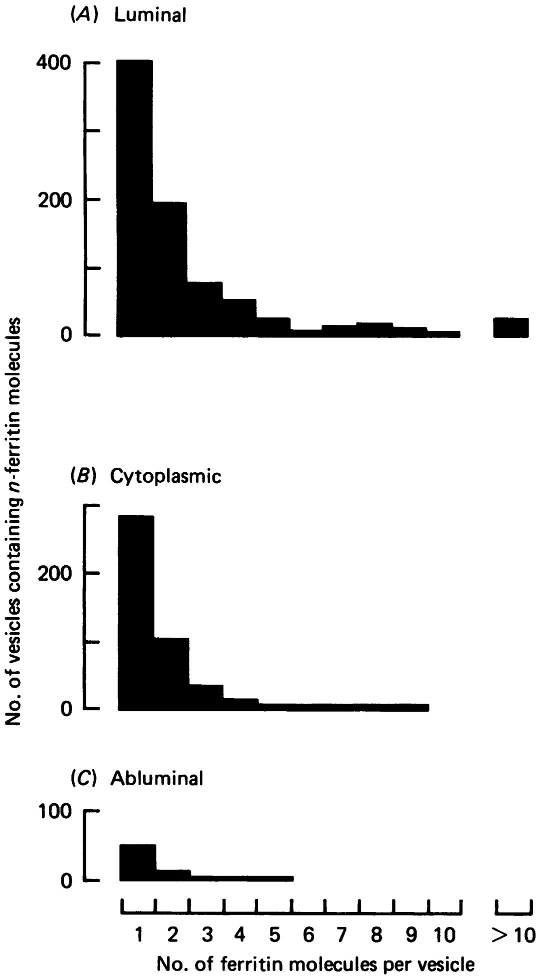

Fig. 6. Distributions of vesicles containing n ferritin molecules in steady-state experiments. A, luminal vesicles (number counter, 1579; number containing no ferritin, 746); B, cytoplasmic vesicles (number counted, 1700; number containing no ferritin, 1253); C, abluminal vesicles (number counted. 680; number containing no ferritin, 617).

and half from the abluminal surface, N_L/N_T for cytoplasmic vesicles should have a minimal value of half N_L/N_T for the luminal vesicles in the steady state. Our data appear to support this prediction since the steady-state values of N_L/N_T in the cytoplasm are approximately half the values for the luminal vesicles. That cytoplasmic N_L/N_T is not much more than the minimal value (and this is particularly true of the experiments where the perfusate contained no albumin), implies that nearly all the ferritin-containing vesicles in the cytoplasm are derived from the luminal surface and very few (if any) vesicles leave the abluminal surface without unloading their ferritin.

A second and perhaps more general prediction of vesicle translocation is that the contents of a cytoplasmic vesicle derived from the luminal surface should be the same as those of an average luminal vesicle. Thus in our experiments the ferritin concentration per labelled cytoplasmic vesicle should be equal to the ferritin concentration per labelled luminal vesicle. Our finding that in the steady state F/N_L for the cytoplasmic vesicles is significantly below its value for the luminal vesicles (Fig. 5) contradicts this important prediction of the translocation hypothesis. Since the translocation hypothesis demands that nearly all cytoplasmic vesicles are derived from luminal vesicles (see above), this discrepancy between our observations of F/N_L and its predictions is serious enough for us to consider another theory.

A possible mechanism, which would account for the lower F/N_L of cytoplasmic in comparison with luminal vesicles, involves the transient fusion of vesicles and mixing of their contents (see Fig. 7). Morphological evidence for the fusion of cytoplasmic vesicles is easy to find in our own data (Plate 1*B–D*) and in electron micrographs of others. In this respect the recent paper of Bundgaard, Frøkjaer-Jensen & Crone (1979) is of particular interest. These authors suggest that all vesicles in the cytoplasm of frog endothelial cells are fused to a neighbouring vesicle and thus their cavities communicate with the extracellular fluid at either the luminal or the abluminal surface. This view is not entirely consistent with the ferritin labelling studies reported in the present paper and it is possible that the large number of communicating vesicles seen by Bundgaard *et al.* is a consequence of their staining technique, which was applied after the tissues had been fixed. Thus the extensive free communications which they describe may be found only in fixed tissues. Whereas it is possible that some of the fusions between vesicles may lead to relatively stable structures, we believe the majority of fusions are short-lived but allow intermixing of the vesicular contents and thus a pathway through the capillary wall for ferritin. Evidence for the existence of a few relatively stable connexions between cytoplasmic vesicles and luminal and abluminal vesicles is suggested by a closer examination of the sequence of labelling.

Labelling after short perfusions: evidence for stable vesicular channels

Figs. 1–3 reveal that some vesicles at sites within the endothelial cell are labelled with ferritin after perfusions of less than 1 sec. With perfusions lasting up to 7 sec, luminal labelling increases progressively whereas labelling of the cytoplasmic and abluminal vesicles remains constant. The luminal vesicles which are not immediately labelled appear unable to pass on their ferritin to cytoplasmic vesicles for 7 sec or more. The latter observation was made by Loudon *et al.* (1979), who suggested ferritin cannot enter vesicles during the last 10 sec of the vesicles' residence at the luminal surface. It is interesting to note that Palade & Bruns (1968) have described morphological differences between the necks of luminal and abluminal vesicles which, they suggest may restrict access during the opening and closing phases of the vesicle's residence at the cell surface. One may imagine that the probability of a luminal vesicle fusing with a cytoplasmic vesicle (of budding from the cell surface) may be increased by a particular distribution of proteins in the membrane and that the development of this configuration increases the diffusion barrier at the vesicle neck, virtually closing it to ferritin (S. Weinbaum, personal communication).

Although this suggestion may account for the failure of cytoplasmic labelling over the first few seconds, it does not account for the constant labelling seen in the cytoplasmic and abluminal vesicles over the first few seconds. To account for this we suggest that a small fraction (*ca.* 10 %) of the cytoplasmic vesicles communicates directly (or through luminal vesicles) with the luminal surface. That there are a few abluminal vesicles labelled in this way indicates that some abluminal vesicles communicate directly with the luminal surface forming transendothelial channels of the type described by Simionescu *et al.* (1975). The relative constancy of N_L/N_T for the cytoplasmic vesicles over the first few seconds of perfusion (Fig. 3) suggests that channels between the cytoplasmic vesicles and the luminal surface are relatively stable structures with a slow turnover. Their numbers are not influenced by the increasing level of luminal labelling, a finding which indicates they are not a phase of every vesicle's life cycle, and which also implies that the early cytoplasmic labelling is less likely to be an artifactual communication produced by the fixative on labelled luminal vesicles. The rapid labelling of a few cytoplasmic vesicles was noted by Karnovsky (1967), who suggested that these were luminal vesicles with necks out of the plane section. We imagine that the rapidly labelling cytoplasmic vesicles are part of the structural picture of intercommunicating vesicles suggested by Bundgaard *et al.* (1979) but we note that their hypothesis would require that 50 % of the cytoplasmic vesicles should be labelled at earliest times. The labelling of abluminal vesicles at early times is very low and more variable than cytoplasmic labelling (Fig. 3). It could be that in addition to a very small number of transendothelial channels, some abluminal vesicles transiently fuse and separate from more stable luminal–cytoplasmic communications. It is interesting that whereas the early cytoplasmic labelling is not significantly affected by the presence of protein in the perfusate, early abluminal labelling is conspicuously increased in its absence.

The availability of luminal vesicles for labelling with ferritin: the effects of albumin

At present we can think of two general explanations for the rise of luminal N_L/N_T over the first 30 sec or so of perfusion. If all the luminal vesicles open through their necks to the lumen of the vessel, as they appear to do, the rise in luminal N_L/N_T could represent the slow diffusion of ferritin through these necks. Alternatively, the neck of the vesicle could be permeable to ferritin for only a fraction of the vesicle's life at the luminal surface. Thus the diffusion of ferritin through an open neck may be complete within 1 sec and the increase in N_L/N_T would represent the increasing number of luminal vesicles passing through the phase of having a permeable neck.

Loudon *et al.* (1979) favoured the diffusion hypothesis on the grounds that at all times their data appeared to be described by a Poisson distribution, which indicated accessibility of all luminal vesicles. A Poisson distribution, however, describes our data less well and the non-steady-state points (particularly those determined in the absence of albumin) fall below the curve in Fig. 4*A*. These deviations, though not statistically significant, are consistent with a fractional of luminal vesicles being unavailable for labelling. This seems reasonable, for in the previous section it was necessary to propose that luminal vesicles become inaccessible during the latter part of their residence at the luminal surface. We therefore favour the view that the early rise in luminal N_L/N_T is the consequence of an increasing fraction of luminal vesicles becoming accessible to luminal ferritin.

If ferritin enters the available vesicles by a random process such as diffusion, then not all the accessible vesicles will necessarily be labelled with ferritin. As previously argued by Loudon *et al.* (1979), the fraction of available vesicles labelled is related to the mean concentration of ferritin present in the available vesicles as determined by a Poisson distribution. The greater the average concentration of ferritin in the available luminal vesicles, the greater the fraction of available vesicles labelled.

If luminal vesicles are inaccessible to ferritin only during the latter part of their residence at the luminal surface, then the initial rise in luminal N_L/N_T represents the increasing fraction of 'old' vesicles which will have been accessible to ferritin at an earlier time. The rate of increase of N_L/N_T depends on: (i) the rate of turnover of luminal vesicles; and (ii) the fraction of luminal vesicles which can be labelled at any time.

The second factor suggests that the initial rate of increase of N_L/N_T should be proportional to its value in the steady state when the turnover rate of labelled luminal vesicles is constant. Evidence relevant to this prediction is offered by a comparison of the rates of N_L/N_T in the presence and absence of albumin. Since neither the diffusion coefficient (see above) nor the relative viscosity of ferritin solutions (Loudon *et al.* 1979) are significantly affected by the presence of albumin, the effect of albumin involves and interaction with the capillary wall. In the presence of albumin the rate of increase of N_L/N_T for luminal vesicles is 0·016 sec^{-1} and in the absence of albumin is 0·024 sec^{-1}. The ratio of these numbers (0·666) is almost the same as the ratio of values for N_L/N_T in the steady state when albumin is present (0·58) and when it is absent (0·90). This agreement supports the hypothesis that the rise in luminal N_L/N_T represents an increasing fraction of luminal vesicles becoming available. It also suggest that the action of albumin is to reduce the volume of distribution of ferritin within the vesicular cavities. Loudon *et al.* (1979) drew attention to the exclusion of ferritin from the cavities of luminal vesicles over and above steric exclusion exerted by the walls of the vesicles. They suggested this additional exclusion might represent the effect of a layer of ruthenium-red-staining material (Luft, 1966; Shirahama & Cohen, 1972) which appears to coat the interior of the vesicle. Our data in Table 2 confirm the degree of exclusion of ferritin from luminal vesicles reported by Loudon *et al.* (1979). Furthermore, they show that exclusion is slightly but significantly reduced when albumin is removed from the perfusate. If the ruthenium-red-staining material is envisaged as a network of glycoprotein chains (the cell coat), albumin could reduce the available space within it by being loosely bound to the chains, so occupying free space within the network, and also possibly drawing the fibres together, i.e. tightening the mesh.

This view is very close to that expressed by Florey (1970). Commenting on the data of Jennings & Florey (1967), he suggested that the presence of plasma proteins in the fluid used to perfuse the isolated rat heart was responsible for the decreased uptake of ferritin by the endothelial cell vesicles, possibly by some interaction with the cell coat described by Luft. In the vessels free from plasma protein the cell coat disrupted, allowing more ferritin to be taken up by the vesicles.

A very different effect of albumin has been described by Wagner, Williams, Matthews & Andrews (1980) using isolated endothelium from rat epididymal fat as a cellular model for vesicular transport. They have presented evidence which suggests that whereas ferritin is taken up by the vesicles, albumin is not. In addition they have shown that ferritin uptake is reduced when albumin is present in the medium bathing the isolated capillaries. These findings suggest that the entry of a macromolecule into the vesicle is a property specific to a particular macromolecule. Our finding that ferritin molecules appear to be randomly distributed within the luminal vesicles under steady-state conditions is inconsistent with their conclusions. It is difficult to see how albumin (molecular radius 3·6 nm) could be excluded from the luminal vesicles when ferritin (molecular radius 6·1 nm) appears to be randomly distributed within them.

This discussion leads us to the picture of endothelial cell vesicles illustrated in Fig. 7. It is thought that only a fraction of the luminal vesicles are accessible to luminal

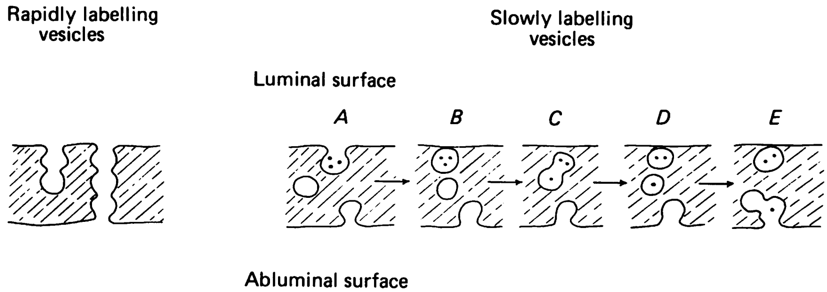

Fig. 7. Hypothesis to account for the entry of ferritin into cytoplasmic and abluminal vesicles of endothelial cells. Two mechanisms are proposed: a small number of rapidly labelling vesicles which have access to the luminal surface via channels formed by the relatively stable fusion of adjacent vesicles (*left*), and slowly labelling vesicles which undergo transient fusion and separation with their neighbours allowing intermixing of vesicular contents (*right A–E*).

ferritin at any instant. After such a period of availability the luminal vesicles become functionally closed to the capillary lumen for 10–30 sec before fusing with cytoplasmic vesicles for a period long enough to allow mixing of the vesicular contents. Separation of the vesicles occurs and subsequent fusion of the cytoplasmic vesicles with other cytoplasmic vesicles and with abluminal vesicles allows plasma macromolecules to pass through the cell. In addition, a small number of cytoplasmic vesicles (10–15 %) communicate directly with the luminal surface forming relatively stable channels. Some chains of fused vesicles might form channels right through the cell (Simionescu *et al.* 1975). Ferritin appears to be excluded from the cavities of vesicles and channels to a greater extent than would be predicted from the steric interaction between ferritin molecules and the vesicle walls. It is suggested that the additional exclusion results from the presence of a glycoprotein network, akin to the cell coat, which lines the vesicles and channels and loosely binds to serum albumin.

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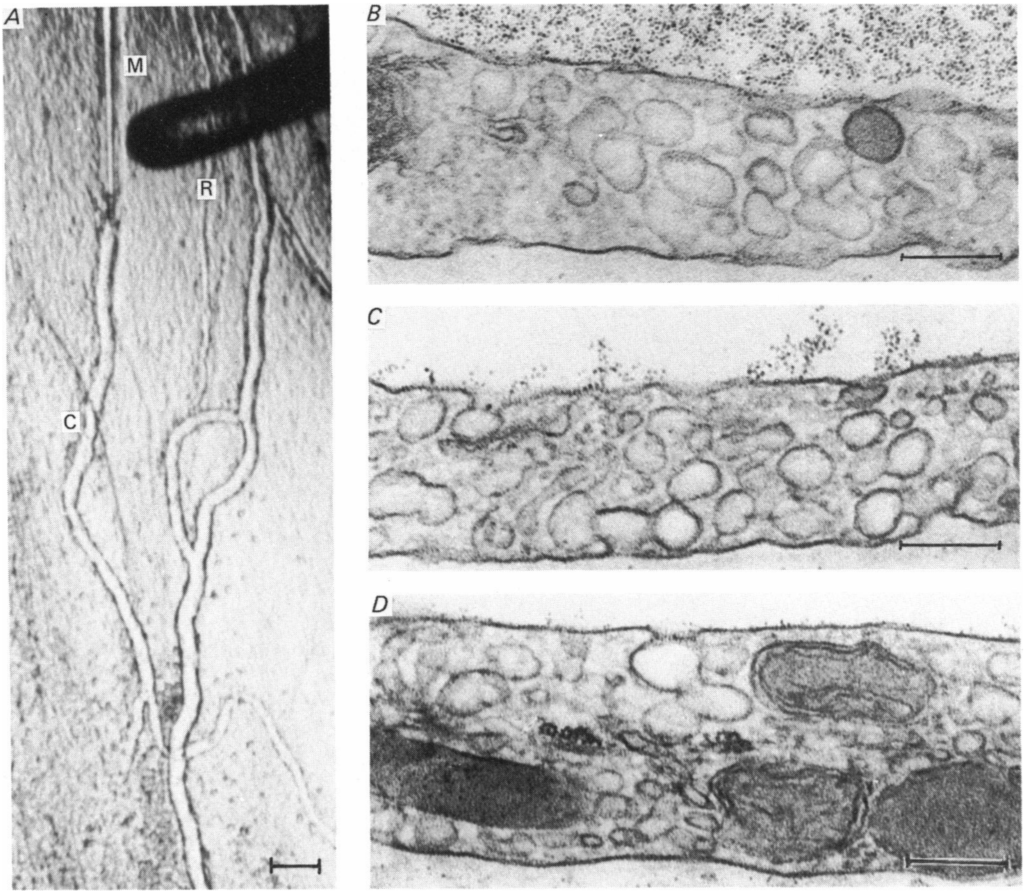
EXPLANATION OF PLATE

A, light micrograph of capillary in frog mesentery perfused with ferritin shortly before fixation *in situ*. M, micropipette; R, restraining microrod; C, perfused capillary; calibration bar, 100 μm. *B–D*, electron micrographs of transverse sections of endothelium of perfused capillaries: *B*, vessel perfused with ferritin and 1 % albumin; *C* and *D*, no albumin in perfusate. Note that a number of vesicles appear to be fused with their neighbours. Calibration bars. 0·2 μm.



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